Luminescence 2000;15:371–376 *ORIGINAL RESEARCH*

Detection of unwanted residues of ivermectin in bovine milk by dissociation-enhanced lanthanide fluoroimmunoassay

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Received 22 June 2000; revised 25 August 2000; accepted 7 September 2000

ABSTRACT: Avermectins are frequently used to control parasitic infestations in many animal species. Previous studies have shown the long-term persistence of unwanted residues of these drugs in animal tissues and fluids. An immunoassay screening test for the detection and quantification of ivermectin residues in bovine milk has been developed. After an extensive extraction procedure, milk samples were applied to a competitive dissociation-enhanced lanthanide fluoroimmunoassay using a monoclonal antibody against an ivermectin–transferrin conjugate. The monoclonal antibody, raised in Balb C mice, showed cross-reactivity with eprinomectin (92%), abamectin (82%) and doramectin (16%). The limit of detection of the assay (mean + 3 SD), calculated from the analysis of 17 known negative samples, was calculated as 4.6 ng/mL. Intra- and inter-assay RSDs were determined as 11.6% and 15.8%, respectively, using a negative bovine milk sample fortified with 25 ng/mL ivermectin. Six Friesian milking cows were treated with ivermectin, three with a pour-on formulation of the drug and three with an injectable solution at the manufacturer's recommended dose rate. An initial mean peak in ivermectin residue concentration was detected at day 4 (mean level = 47.5 ng/mL) and day 5 post-treatment (mean level = 26.4 ng/mL) with the injectable form and pour-on treatment, respectively. A second peak in residue concentration was observed using the DELFIA® procedure 28 days post-treatment in both treatment groups (23.1 ng/mL injectable and 51.9 ng/mL pour-on). These second peaks were not confirmed by HPLC and must at this time be considered to be false-positive results. By day 35 after treatment the mean ivermectin residue concentration of both groups fell below the limit of detection of the assay.

KEYWORDS: ivermectin; avermectins; milk; residues; fluoroimmunoassay

INTRODUCTION

Ivermectin is the most commonly used member of a group of macrocyclic lactone anthelminthics known as the avermectins. It is naturally occurring and has proved extremely useful in veterinary medicine due to its broad-spectrum activity against both helminths and arthropods (1).

Since its introduction into farming practice, many workers have studied the pharmacokinetic disposition of ivermectin in farm animal species (2, 3, 4). During these studies, ivermectin has been shown to be highly lipophilic, resulting in long-term persistence of residues in both animal tissues and fluids. Due to the popularity of ivermectin as an anthelminthic treatment and the production of MRLs for this and other avermectin drugs, a large number of different methods have been employed to detect their residues in animal tissue. Initially, method development focused largely on HPLC-based procedures, such as that described by Tway *et al.* (5); however, more recently a number of alternative techniques have been used, including immunoassay (6).

Although not recommended for use in lactating animals, pharmacokinetic studies have shown that milk taken from a number of species of treated animals shows long-term persistence of the drug residues (7, 8). Due to this persistence, it is important that regulatory bodies have available methods suitable for residue detection in this matrix. To date, only a limited number of methods, based largely on HPLC, have been described for use with milk (8, 9).

The aim of this study was to develop an immunoassay procedure capable of detecting ivermectin residues in milk samples, which could be applied in a routine surveillance programme.

The dissociation-enhanced lanthanide fluoroimmmunoassay (DELFIA®) was used to determine ivermectin residue concentrations in milk samples taken from six cows over a 35 day period after treatment with both pour-on and injectable ivermectin formulations at the recommended therapeutic rates.

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MATERIALS AND METHODS

Reagents

The europium labelling reagent and DELFIA® enhancement solution were obtained from EG & G Wallac (Crownhill, Milton Keynes). Ivermectin reference standard was purchased from Sigma; the remaining standards were kindly supplied as gifts. Abamectin and eprinomectin were suppied by Merck (Rahway, NJ) while doramectin was supplied by Pfizer (Louvain-la-Neuve, Belgium). All other chemicals or reagents were purchased from Sigma (Poole, Dorset).

Buffers and solutions

Antibody coating solution contained 0.009% sodium chloride in 50 mmol/L dipotassium hydrogen orthophosphate solution. Blocking buffer contained 0.03% Trehalose, 0.001% bovine serum albumin and 0.001% Germall II in 20 mmol/L Tris–HCl, pH 7.2. Assay buffer contained 0.0001% Tween 20, 0.009% sodium chloride, 0.0005% bovine serum albumin and 0.0005% sodium azide in 50 mmol/L Tris–HCl, pH 7.75.

Antisera production

A monoclonal antibody was raised to an ivermectin transferrin immunogen. 5-*O*-Succinoylivermectin was prepared as described previously (6) and conjugation to the carrier protein achieved by carbodiimide activation (10). Balb C mice were inoculated with the immunogen at weeks 0, 3, 16, 17 and 18 and the polyclonal response monitored. The mouse showing the highest polyclonal titre was killed and the spleen fused with a mouse myeloma (11). Of 820 resultant hybridomas screened, 70 showed evidence of secreting antibodies to ivermectin. The most promising of these was cloned twice prior to ascites production. Ascites was purified using HiTrap Protein G columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) prior to storage at −80°C until required.

Europium labelling of ivermectin conjugate

An ivermectin–human serum albumin (HSA) conjugate was prepared using the 5-*O*-succinoylivermectin derivative by carbodiimide activation. Europium labelling of the conjugate was performed using a labelling kit, as described in the manufacturers' instructions. Briefly, 0.2 mg europium in 0.1 mol/L carbonate-bicarbonate buffer, pH 9.8, was added to 1 mg protein (HSA conjugated to ivermectin) in 0.1 mol/L carbonate–bicarbonate buffer, pH 9.8. The mixture was incubated overnight at room temperature, then separation of labelled protein from free europium achieved by gel filtration on a fast desalting fast protein liquid chromatography (FPLC) column (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Extraction procedure

The milk extraction procedure was a modified version of that employed by Crooks *et al.* (6) in the extraction of ivermectin from bovine liver. Briefly, milk samples (4 mL) were extracted into acetonitrile and the extract washed with hexane. The acetonitrile layer was evaporated to dryness and the residue resuspended in ethyl acetate. The ethyl acetate was applied to a NH_2 solid phase extraction (spe) column, the eluate collected and evaporated to dryness. The residue was resuspended in assay buffer for use in the DELFIA® procedure.

Immunoassay procedure

Antimouse IgG microtitration strips (EG & G Wallac, Turku, Finland) were washed once using an isotonic saline solution containing 0.0125% Tween 20 detergent. The antibody was diluted 1 + 1999 in coating solution and immobilized on the wells of the microtitration strips by the addition of 100 μL to each well. The microtitration strips were covered, then incubated overnight at room temperature. Excess coating solution was removed by tapping gently on absorbent paper and non-specific binding decreased by the addition of 150 μL per well of blocking buffer, and incubation for 1 h at room temperature (RT). Immediately prior to use, blocking buffer was removed by washing once with wash solution.

Aliquots (50 μL) of the standard, control and sample extracts were added to the appropriate wells in duplicate. To all wells, 50 μL assay buffer containing the ivermectin–europium conjugate, diluted 1 + 9999, was added.

The microtitration strips were incubated for 2 h at room temperature on a plate shaker to allow equilibrium to be reached, after which excess reagents were removed by an extensive wash cycle. After this, 150 μL enhancement solution was added to each well and the plate incubated for 10 min at RT on a plate shaker.

Fluorescence in each well was measured using a Victor 1420 multilabel counter (Wallac Oy, Turku, Finland). Calibration curves were constructed from the incorporated standards allowing calculation of ivermectin concentration in unknowns.

EXPERIMENTAL

Animal medication

A group of seven Friesian cows, which form part of the strictly controlled herd belonging to this institute, were used during the study. These animals were known to be

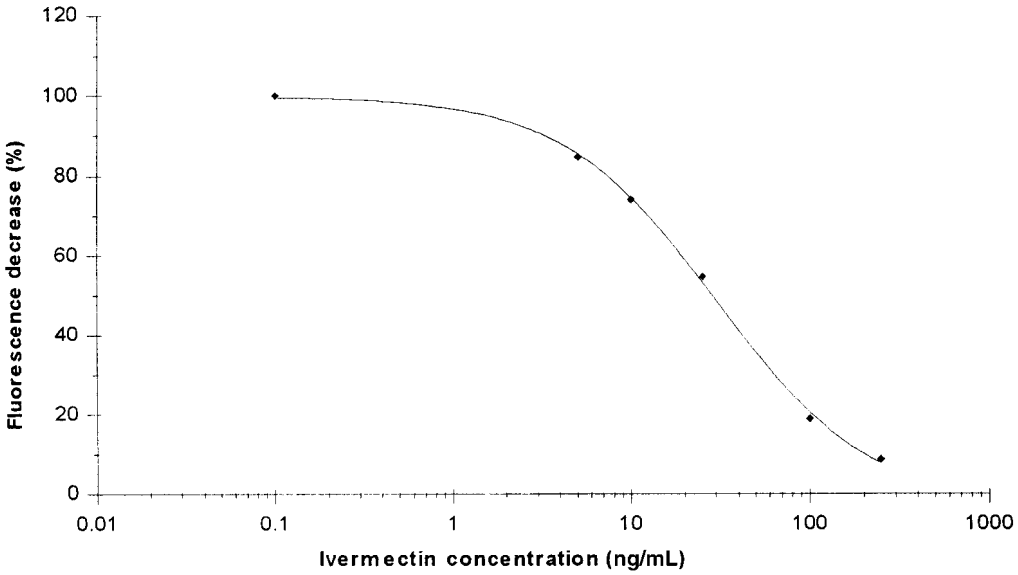

Figure 1. Typical calibration curve after extraction from milk.

free from treatment with any avermectin formulation. For the purposes of the trial, the seven cows, with a mean body mass of 545 kg, were randomly divided into three groups. Groups 1 and 2 consisted of three cows each; the seventh animal remained untreated for the duration of the experiment and acted as a control.

Group 1 animals were treated with a pour-on formulation of ivermectin (Ivomec) at the recommended dose rate of 500 μg/kg bodyweight (this equated to 1 mL of the formulation per 10 kg bodyweight). The animals were treated when the hair and hide of each was dry and were housed in a separate byre for 2 h post-treatment to ensure that they were neither exposed to rain nor in contact with the remaining four animals. Group 2 cows were treated with an injectable form of ivermectin (Panomec) at a dose rate of 200 μg/kg bodyweight (this equated to 1 mL of the formulation per 50 kg bodyweight). After treatment, the seven cows were returned to the herd for the duration of the trial.

Animal sampling

Milk samples were taken by hand twice daily (morning and evening) from all experimental animals for a period of 7 days post-treatment. Samples were taken once per week for a further 4 week period. The samples were taken from all quarters by hand mid-milking. The quarter milk samples taken from each cow were pooled to provide a daily sample for each animal and were stored at −20°C until analysis. Milk samples taken from one animal in each group (days 0–5 after treatment) were analysed by an HPLC procedure (12). Further HPLC analysis was performed on milks taken from these animals on days 7, 14, 21, 28 and 35 post-treatment.

RESULTS AND DISCUSSION

The specificity of the antisera raised was estimated using the procedure outlined by McCaughey *et al.* (13). Significant cross-reactivity was found with other avermectins tested: eprinomectin (92%), abamectin (82%) and doramectin (16%). No significant cross-reactivity was found with the milbemycin moxidectin or any of the other anthelminthic compounds analysed.

Fig. 1 shows a typical calibration curve for ivermectin using the DELFIA® procedure. The working range of the assay was determined as 1.25–62.5 ng/well (5–250 ng/mL). The mean mid-point of the curve was calculated as 8.4 ng/well (33.5 ng/mL).

Tables 1 and 2 outline the intra- and inter-assay variation calculated for the milk assay. Analysis of 17

Table 1. Intra-assay parameters determined for the ivermectin milk dissociation-enhanced lanthanide fluoroimmunoassay

Replicates (*n*)	Expected (ng/mL)	Observed (ng/mL)	Standard deviation (ng/mL)	RSD (%)
17	0	1.98	0.86	
10	25	20.28	3.21	15.8
10	125	139.94	15.87	11.3

Table 2. Inter-assay parameters determined for the ivermectin milk dissociation-enhanced lanthanide fluoroimmunoassay

Replicates (*n*)	Expected (ng/mL)	Observed (ng/mL)	Standard deviation (ng/mL)	RSD (%)
6	25	22.26	2.58	11.6
6	125	118.68	10.57	9.6

Table 3. Concentrations of ivermectin (ng/mL) detected by fluoroimmunoassay and high performance liquid chromatography in the milk of experimental animals treated with a pour-on formulation of the drug

Days post-treatment	Animal No.			
	1 (DELFIA®)	1 (HPLC)	2 (DELFIA®)	3 (DELFIA®)
0.5	5.8	0.0	8.3	0.7
1	10.4	8.2	15.0	19.5
2	5.3	16.5	26.7	31.9
3	8.9	13.2	22.1	23.3
4	8.9		18.8	21.4
5	66.8	3.7	11.4	1.0
6	17.1		0.0	0.0
7	8.1	6.1	0.0	0.0
14	0.0	1.6	10.2	6.0
21	0.0	0.0	7.0	8.9
28	89.7	0.0	31.6	34.5
35	0.0	0.0	3.1	6.6

milks known to be avermectin-free gave values of 1.98 ± 0.86 ng/mL. The limits of detection (mean $\pm$ 3 SD) and determination (mean $\pm$ 6 SD) of the assay were calculated as 4.6 and 7.1 ng/mL, respectively.

The concentration of ivermectin residues detected in the milk samples obtained during the experimental period showed pronounced animal-to-animal variation. This effect was more notable in the animals treated with the pour-on formulation of the drug. In the pour-on group (Table 3), the mean concentration of drug detected by DELFIA® post-treatment remains fairly consistent from day 2 to day 5 (range 16–26 ng/mL). From day 6 to day 21, the concentrations detected are substantially lower (range 3–6 ng/mL). On day 28 of sampling, a mean level of drug residue was detected in excess of all other measurements made during the experiment (mean 52 ng/mL). This surge in detectable immunoreactive residue was apparent in all three animals in this group. One week later the mean residue concentration was below the limit of detection of the assay.

In the injectable group (Table 4) the mean amounts of residue present in the milk samples were 12–48 ng/mL over the course of the first 4 days of withdrawal. After this time, mean concentrations were in the range 6–10 ng/

Table 4. Concentrations of Ivermectin (ng/mL) detected by fluoroimmunoassay and high performance liquid chromatography in the milk of experimental animals treated with an injectable formulation of the drug

Days post-treatment	Animal No.			
	4 (DELFIA®)	5 (DELFIA®)	6 (DELFIA®)	6 (HPLC)
0.5	14.7	16.8	4.4	0.0
1	35.7	29.0	6.7	11.9
2	25.4	100.0	15.0	24.4
3	33.1	36.1	27.4	17.4
4	27.7	32.2	82.7	16.1
5	0.0	0.0	17.2	
6	6.7	0.0	11.6	
7	15.8	3.3	9.9	11.6
14	7.4	8.6	8.7	5.7
21	2.2	4.2	61.3	0.0
28	22.0	22.3	25.0	0.0
35	6.5	0.0	3.8	0.0

mL until days 21 and 28 post-treatment, when elevated concentrations were once again observed. By the end of the sampling period (35 days after treatment) the mean residue level was below the limit of detection. Milk taken from the control animal remained lower than the limit of detection of the method throughout the trial.

The HPLC procedure, which employed similar sample preparation to the DELFIA® clean-up, was performed on a limited number of the experimental samples, showing some interesting comparisons. There was general agreement between the DELFIA® results and HPLC during the early sampling period. However, the secondary rise in residue concentrations measured by DELFIA® was not detected by the HPLC procedure.

The present study describes for the first time the application of an ivermectin monoclonal antibody to drug residue screening. Previously, Schmidt *et al.* (14) described monoclonal production to the avermectins but did not apply the resultant antibodies to sample analysis. The antibody produced in this study showed significant cross-reactivity with other members of the avermectin family, including eprinomectin, the only member licensed for use in lactating cattle. There is therefore potential to exploit this antibody in the development of a more 'multi-residue'-orientated screening procedure for the family of avermectin compounds. The sensitivity of the assay (limit of detection 4.6 ng/mL) was considered adequate for screening purposes. Although it was difficult to determine how sensitive such screening tests should be, as no MRL exists for ivermectin in milk, the EU MRL for eprinomectin (20 ng/mL) was used as a guideline for determining a target sensitivity. Intra- and inter-assay RSDs for the developed test fell within acceptable limits for immunoassay procedures and were similar to those determined for other enzyme immunoassay (6, 15) and fluoroimmunoassay (16) procedures used within this laboratory.

Analysis of ivermectin residues in the milk taken from treated cows gave rise to some unexpected results. A previous study (17) using HPLC analysis recorded a single phase elimination of ivermectin residues in milk following animal treatment. In the present study, a secondary phase of elimination, observed in all experimental animals ($n = 6$), occurred at day 28 post-treatment as measured by DELFIA® but not by HPLC. The mean ivermectin concentrations detected by DELFIA® in the milk taken from the cows treated with the injectable form of the drug (47.5 ng/mL after 4 days) is similar to those reported in previous studies using the same dose and inoculation route. Toutain *et al.* (17), using HPLC, found peak milk concentrations of 40.5 ng/mL within a few days of administration, while Alvernie *et al.* (8) observed higher peak concentrations at 75 ng/mL on day 8 after treatment. Data pertaining to milk residues following pour-on treatment have not previously been reported.

The apparent presence, within this study, of ivermectin residues in milk samples taken 4 weeks after administration cannot easily be explained. The evidence points to the sudden release of an immunoreactive ivermectin metabolite from some biological reservoir. A possible cause for this release may be explained by the management of the farm animals used in this study. For the first 3 weeks of the sampling period the animals were housed indoors and fed on a combination of concentrates and silage. After this time the animals were moved outdoors to pasture. It may be that the change in diet had an influence upon the release of a bound ivermectin component. Tolan *et al.* (18) previously described the binding of avermectins to plasma components and the difficulties in achieving complete extraction of the drug from plasma. Another possible explanation for the presence of detectable drug residue at this stage is the presence of ivermectin contamination in the pasture, as animals medicated with the drug had previously been grazed there. This second possible cause would, however, appear to be unlikely, as the control animal used in the experiment, although also grazed in the pasture, did not excrete any detectable concentration of ivermectin residue in milk.

The assay described appears to provide a relatively simple and sensitive means for use in a routine drug residue screening programme. The assay format allows the results of up to 14 milk samples to be obtained within 24 h of the start of sample preparation. A more rapid analytical time or demand for a higher throughput would require further simplification of the sample extraction procedure to be developed. The effect ivermectin residues may have on milk is as yet unclear and two main aspects of this remain to be investigated. The first and most important is the fact that residues of a widely used drug can be found at concentrations which may well be in excess of a future MRL. The second issue is the effect that this drug may have on the organoleptic properties of products such as cheese and yoghurt, where fermentation processes are involved.

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